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Contrasting growth response of an N_2-fixing and non-fixing forb to elevated CO_2: dependence on soil N supply

Tali D. Lee[1,3,4], Mark G. Tjoelker[1], Peter B. Reich[1] & Michael P. Russelle[2]
[1]*Department of Forest Resources, University of Minnesota, 1530 Cleveland Ave. N., St. Paul, MN 55108, USA.* [2]*USDA-Agricultural Research Service and Department of Soil, Water, and Climate, University of Minnesota, St. Paul, MN, 55108, USA.* [3]*Department of Biology, University of Wisconsin – Eau Claire, Eau Claire, WI 54701, USA.* [4]*Corresponding author**

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Abstract

With the ability to symbiotically fix atmospheric N_2, legumes may lack the N-limitations thought to constrain plant response to elevated concentrations of atmospheric CO_2. The growth and photosynthetic responses of two perennial grassland species were compared to test the hypotheses that (1) the CO_2 response of wild species is limited at low N availability, (2) legumes respond to a greater extent than non-fixing forbs to elevated CO_2, and (3) elevated CO_2 stimulates symbiotic N_2 fixation, resulting in an increased amount of N derived from the atmosphere. This study investigated the effects of atmospheric CO_2 concentration (365 and 700 μmol mol^{-1}) and N addition on whole plant growth and C and N acquisition in an N_2-fixing legume (*Lupinus perennis*) and a non-fixing forb (*Achillea millefolium*) in controlled-chamber environments. To evaluate the effects of a wide range of N availability on the CO_2 response, we incorporated six levels of soil N addition starting with native field soil inherently low in N (field soil + 0, 4, 8, 12, 16, or 20 g N m^{-2} yr^{-1}). Whole plant growth, leaf net photosynthetic rates (A), and the proportion of N derived from N_2 fixation were determined in plants grown from seed over one growing season. Both species increased growth with CO_2enrichment, but this response was mediated by N supply only for the non-fixer, *Achillea*. Its response depended on mineral N supply as growth enhancements under elevated CO_2 increased from 0% in low N soil to +25% at the higher levels of N addition. In contrast, *Lupinus* plants had 80% greater biomass under elevated CO_2 regardless of N treatment. Although partial photosynthetic acclimation to CO_2 enrichment occurred, both species maintained comparably higher A in elevated compared to ambient CO_2 (+38%). N addition facilitated increased A in *Achillea*, however, in neither species did additional N availability affect the acclimation response of A to CO_2. Elevated CO_2 increased plant total N yield by 57% in *Lupinus* but had no effect on *Achillea*. The increased N in *Lupinus* came from symbiotic N_2 fixation, which resulted in a 47% greater proportion of N derived from fixation relative to other sources of N. These results suggest that compared to non-fixing forbs, N_2-fixers exhibit positive photosynthetic and growth responses to increased atmospheric CO_2 that are independent of soil N supply. The enhanced amount of N derived from N_2 fixation under elevated CO_2 presumably helps meet the increased N demand in N_2-fixing species. This response may lead to modified roles of N_2-fixers and N_2-fixer/non-fixer species interactions in grassland communities, especially those that are inherently N-poor, under projected rising atmospheric CO_2.

Introduction

Rising atmospheric carbon dioxide (CO_2) concentration and increasing inputs of fixed forms of nitrogen (N) into the global N cycle are predicted to have profound effects on ecosystems (Vitousek et al., 1997). While numerous studies document the response of plants to these factors independently, fewer evaluate

* FAX No: 715-836-5089.
E-mail: leetd@uwec.edu

the combined effects of elevated CO_2 and increased soil N supply, which likely interact in complex ways and differently at different scales. Plant responses to elevated CO_2 are fundamentally mediated by photosynthesis (Drake et al., 1997) and a suite of physiological, morphological and growth changes (Curtis and Wang, 1998). Typical increases in photosynthetic rates and biomass accumulation in elevated compared to current ambient CO_2 concentrations have ranged between 20 and 50% in crops, with responses of wild species in natural systems being more highly variable, and often considerably lower in magnitude (Poorter, 1993; Wand et al., 1999; Ward and Strain, 1999).

The variation in growth and photosynthetic enhancements under elevated CO_2 may be associated with the differential responses of species to other limiting resources such as nutrients, water, and light (Drake et al., 1997; Hebeisen et al., 1997a,b; Lloyd and Farquhar, 1996; Soussana and Harwig, 1996). Since available N already limits productivity in most ecosystems, and because tissue N is a major determinant of photosynthesis (Reich et al., 1997), low N may reduce potential photosynthetic rate and growth enhancements under elevated CO_2, and thus limit the ability to incorporate additional carbon (Drake et al., 1997; Poorter, 1993). Some simulation models suggest that growth responses to elevated CO_2 concentrations are constrained by N limitation (Rastetter et al., 1997), although actual evidence is mixed (Poorter, 1998). Experimental results range from no consistent effect of nutrient availability on plant responsiveness to elevated CO_2 (Lloyd and Farquhar, 1996; Reich et al., 2001b) to a decreased CO_2 sensitivity that is linked to low nutrient availability (Curtis and Wang, 1998; Leadley and Körner, 1996; Poorter, 1998). Much of the uncertainty that hinders our ability to predict responses of vegetation to elevated CO_2 is related to our lack of understanding of the nature of N limitations (Vitousek and Field, 1999).

If the CO_2 response is N-limited, then legumes should demonstrate greater and more sustained responses to elevated CO_2 than non-fixers due to the ability to fix atmospheric N_2, and therefore modulate their own N supply. N_2-fixing species often show larger growth responses to elevated CO_2 than non-fixing species (Hebeisen et al., 1997a,b; Lüscher et al., 1996, 2000; Poorter, 1993; Soussana and Hartwig, 1996). However, other studies do not show consistently greater responses in legume species compared to non-legumes (Leadley and Körner, 1996; Reich et al., 2001b), perhaps owing to growth on soil deficient in other resources (P, K, and H_2O) required for optimal N_2 fixation (Almeida et al., 2000; Bordeleau and Prévost, 1994; Lüscher et al., 1996; Niklaus et al., 1998).

Symbiotic N_2 fixation is an important source of N in many ecosystems and is thought to be crucial for helping meet N demands in grassland systems, especially those exposed to elevated CO_2 where growth and N demand are presumed to be greater (Lüscher et al., 2000; Zanetti and Harwig, 1997). Increases in nitrogenase activity within the nodules of legumes can occur within several days of exposure to elevated CO_2 (Murphy, 1986). However, less is known concerning the effects of CO_2 on the relative contribution of symbiotically fixed N_2 to total N in plants or ecosystems over the long term. Recent studies using ^{15}N methods have found increases in the amount of N derived from symbiotic N_2 fixation that coincide with increases in legume plant growth at elevated CO_2 (Soussana and Hartwig, 1996,; Zanetti et al., 1996, 1998; Zanetti and Harwig, 1997).

Our objective was to consider the role of symbiotic N_2 fixation in the CO_2 response and to evaluate potential interactive plant responses to elevated atmospheric CO_2 and enriched N addition by combining measures of net photosynthesis and whole plant growth with estimates of N derived from symbiotic N_2 fixation in N_2-fixing and non-fixing species. The present study extends prior research from predominately managed systems to wild perennial species that co-occur naturally in tallgrass prairie plant communities. We compared the growth and photosynthetic responses of *L. perennis* (N_2-fixing legume) with *A. millefolium* (non-fixing forb) to elevated CO_2 concentrations across a range of soil N availability. We tested the hypotheses that (1) the CO_2 response of wild species is limited at low N availability, (2) legumes respond to a greater extent than non-fixing forbs to elevated CO_2, and (3) elevated CO_2 stimulates symbiotic N_2 fixation resulting in an increased amount of N derived from the atmosphere.

Materials and methods

Plant material and soil

We obtained seeds of wild lupine (*Lupinus perennis* L.) and yarrow (*Achillea millefolium* L.) originating from populations from the upper Midwest, USA (Prairie Restorations, Inc., Princeton, MN). Species

hereafter are referenced by their genus. Ten seeds were sown in 2600 cm^3 pots (one species per pot: 10.2 cm diameter, 32 cm height, polyvinyl chloride) filled with sandy soil collected from grassland fields located at Cedar Creek Natural History Area, Minnesota, a location at which these species are commonly found. The soils are classified as entisols derived from a glacial outwash sandplain, 94% sand, classified in the Nymore sand series, acidic (pH = 5.3), and nitrogen poor (total soil N = 0.04%). Mean *Achillea* seed mass was 0.2 mg and *Lupinus* was 20.4 mg. Immediately after sowing, 72 pots (six of each species/treatment combination) were placed in each of four growth chambers (Conviron, E15, Controlled Environments, Inc., Winnipeg, Manitoba, Canada) for a total of 288 pots. Seeds were germinated under uniform conditions of 360 μmol mol^{-1} CO_2, 15-h photoperiod, light/dark temperatures of 25/20 °C, and light/dark relative humidity of 60/80%.

Growth conditions: Carbon dioxide and ^{15}N treatments

CO_2 treatments commenced the third day after sowing when 91% of the pots had at least one germinant. We selected treatment levels of current ambient atmospheric CO_2 concentrations of 365 μmol mol^{-1} and 700 μmol mol^{-1} (approximately 2x ambient) replicated twice among four identical growth chambers. Chambers were programmed to mimic day length at Cedar Creek Natural History Area (Lat. 45° N), corresponding to May 20 – July 30. In each chamber, lighting was provided by two 1000 W metal halide and two 1000 W sodium high-intensity-discharge lamps in a light bank that was raised or lowered throughout the experiment to supply irradiance of approximately 1100 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at average plant height. Photoperiod was divided into three equal periods, two at ca. 600 μmol m^{-2} s^{-1} PPFD at the beginning and end of each photoperiod with the middle period at ca.1100 μmol m^{-2} s^{-1}, for an approximate total of 45 mol $m^{-2}d^{-1}$, representative of integrated solar radiation at midsummer on a clear day. *Lupinus* was inoculated with rhizobia species specific to *Lupinus* (Prairie Moon Nursery, Winona, MN) at the first sign of true leaf emergence by applying 30 mg inoculum powder to the soil surface of each pot followed by water. At this time, seedlings of both species were thinned at random until an individual remained in each pot. Light intensity, light /dark period temperature (25/20 °C) and relative humidity (60/80%), and CO_2 concentrations were monitored regularly to verify treatment conditions. The pots, initially randomly located, were periodically repositioned to randomize any effect of position across all pots within a chamber.

Each chamber assigned the elevated treatment was supplied with CO_2 from its own gas cylinder and concentrations in all chambers were monitored with separate gas analyzers (APBA-250E, Horiba Instruments, Inc., Irvine, CA, USA). Hourly average, maximum, and minimum CO_2 concentrations, based on 1-minute mean values, were recorded with dataloggers (LI-1000, LI-COR, Inc., Lincoln, NE, USA). Average hourly CO_2 concentrations were 365 μmol mol^{-1} (363, 369 μmol mol^{-1}; 95% confidence interval) across the two ambient CO_2 chambers, hereafter designated 365, and 697 μmol mol^{-1} (696, 698 μmol mol^{-1}; 95% confidence interval) in the elevated CO_2 chambers, hereafter designated 700 μmol mol^{-1}.

Soil N treatments were calculated so that additions over the 56 days of the study were equivalent to rates of 0, 4, 8 12, 16, or 20 g N m^{-2} yr^{-1} based on a 12 week growing season. N treatments were assigned to a random selection of six pots per species/treatment combination within each chamber. Applications were made every third day as a solution of ^{15}N-enriched NH_4NO_3 with ammonium and nitrate equally labeled at 5.7 atom% ^{15}N (Isotec, Inc., Miamisburg, Ohio). N was applied in solution at the following concentrations: 0, 27, 55, 82, 109, and 136 g N L^{-1} for total amounts of N applied per pot (≈3.3 kg soil dry mass) equaling: 0, 23, 46, 69, 92, and 116 mg N pot^{-1} over the course of the experiment. No other nutrients were added. The base soil used in this experiment, without amendment, has supplied on average 2.3 g N m^{-2} y^{-1} (P. Reich, unpublished data). Initial soil pH was 5.3, Bray-extractable P was 61 mg P kg^{-1} soil, and 1*M* ammonium acetate-extractable cations were 51 mg K kg^{-1} soil, 328 mg Ca kg^{-1} soil and 50 mg Mg kg^{-1}soil. Final harvest was at 56 days since sowing when individuals began to develop flowers.

Leaf gas exchange and whole plant sampling

In situ rates of leaf net photosynthesis (*A*) were determined every 8–10 days once plants had several fully developed leaves, resulting in four (*Lupinus*) or three (*Achillea*) separate measurement time points until final harvest. Gas exchange was measured on the same plants using a different leaf of similar ontogenetic stage each time to control for variation in leaf traits

due to age. We used upper fully expanded young to mid-aged leaves when leaf traits are relatively stable (Reich et al., 1991). Rates of gas exchange were measured using CIRAS-1 portable infrared gas exchange systems (PP Systems, Hitchin UK) operated in open-configuration controlling temperature, CO_2 concentration, and vapor pressure. Measurements were made typically on ten plants per treatment combination, over a 3-h interval during the period of full irradiance between 8 and 3 pm. Due to time constraints, gas exchange was measured on *Achillea* plants under the 0 and 16 g N m^{-2} yr^{-1} addition levels only, with these two levels chosen to compare *Achillea* with *Lupinus* responses under ambient soil N and a contrasting relatively high N addition level. Rates were determined at or near light-saturating conditions (mean photosynthetically active radiation ± SE: 710 ± 2 μmol m^{-2} s^{-1}, photosynthetic light response data not shown), at 27.2 ± 0.1 °C, and near ambient humidity (mean leaf chamber vapor pressure deficit ± SE: 1.95 ± 0.02 kPa).

Net photosynthetic rates were measured on each sample leaf of *Lupinus* and *Achillea* plants grown and measured in ambient (A_{365}) and elevated CO_2 concentrations (A_{700}). Ambient-grown plants were also measured at elevated CO_2 (700 μmol mol^{-1}) to assess the response of photosynthesis to instantaneous CO_2 enrichment and to compare of rates of plants from both CO_2 treatments at a common CO_2 concentration [A_{700} vs. A_{365}(measured at 700)]. Following gas exchange measurements, leaves were harvested and leaf area was measured using a digital image analysis program (WinRhizo 3.9, Regent Instruments, Quebec). These measurement leaves, as well as whole plants following final harvest, were oven dried (65 °C for 48–72 h) prior to biomass determination.

Tissue N and calculation of N derived from symbiotic N_2 fixation

All dried plant material was finely ground to determine tissue N concentrations and the atom% of ^{15}N (Europa Scientific Integra isotope ratio mass spectrometer, University of California at Davis, Stable Isotope Facility, Davis, CA). The application of N fertilizer with 5.7 atom% of ^{15}N, which is well above the natural concentrations found in the atmosphere, allowed the estimation of the fraction of N derived from fixation (Equation 1, $[\text{proportion N}]_{\text{fixation}}$) based on the ^{15}N-dilution method (Danso et al., 1993; McAuliffe et al., 1958), and the estimation of the amount of N derived fertilizer (Equation 2, $[\text{proportion N}]_{\text{fertilizer}}$)(Blumenthal and Russelle, 1996):

$$(\text{Proportion N})_{\text{fixation}} = 1 - \left[\frac{(a\%^{15}N_{\text{sample plant}} - a\%^{15}N_{\text{background}})}{(a\%^{15}N_{\text{control plant}} - a\%^{15}N_{\text{background}})}\right] \quad (1)$$

$$(\text{Proportion N})_{\text{fertilizer}} = \left[\frac{(a\%^{15}N_{\text{sample plant}} - a\%^{15}N_{\text{control plant}})}{(a\%^{15}N_{\text{label}} - a\%^{15}N_{\text{control plant}})}\right], \quad (2)$$

where $a\%^{15}N$ is the atom% ^{15}N in the plant tissue or the label applied as fertilizer. The $a\%^{15}N_{\text{control plant}}$ values were the mean ^{15}N concentrations of ambient and elevated CO_2 grown control *Achillea* plants (grown without N addition), which were 0.3859 and 0.3867%, respectively. $a\%^{15}N_{\text{background}}$ is the atom% ^{15}N of atmospheric N_2 (0.3663%). This approach integrates symbiotic N_2 fixation with whole plant growth over the growth interval. *Achillea* as the reference plant has presumably similar enough growth patterns, growing period, and rooting depths and distribution to serve as an adequate reference plant for *Lupinus* (Danso et al., 1993). This is supported by data from *Achillea* and *Lupinus*, which show similar functional traits when compared to over 30 other species all growing in the greenhouse and in field monocultures in similar soil in other studies taking place at Cedar Creek Natural History Area, MN (Craine et al., 2002; Reich et al., 2003).

Data analysis

The experimental design was a completely randomized split-plot arrangement with CO_2 concentration as the whole-plot factor using four identical growth chambers, two at each CO_2 concentration. The subplot factor of N addition levels was randomly assigned and replicated in individual pots among the chambers. In ANOVA, all treatment effects were considered fixed. Using *F*-tests, the effect of CO_2 treatment (1 d.f.) was tested against the random effect of chamber nested within CO_2 (2 d.f.). The main effect of N addition (5 d.f.) and the CO_2*N interaction were tested against the residual error (13 d.f.). The main effect of N was partitioned into single-degree-of-freedom contrasts for both linear (*l*) and quadratic (*q*) terms to examine the response to N levels (Sokal and Rohlf, 1995). Species were analyzed separately unless otherwise noted. All analyses were conducted with statistical analysis software (JMP Version 3.2.6, SAS Institute

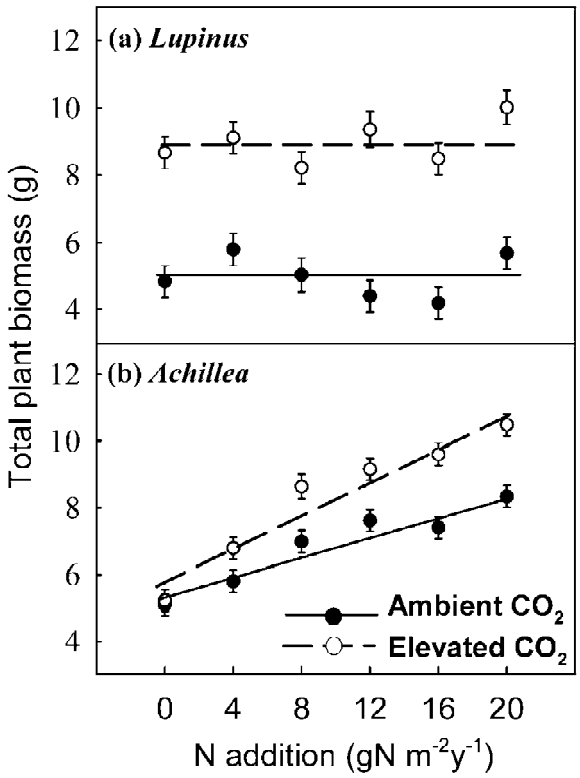

Figure 1. Total plant biomass (g) of (a) *Lupinus* and (b) *Achillea* plants after 56 days of growth under ambient (•, 365 μmol mol^{-1}) and elevated (◯, 700 μmol mol^{-1}) CO_2 and varying levels of N addition. Least squares means (±SE, $n = 8 - 10$) are plotted with CO_2 treatment mean lines for *Lupinus* and CO_2*N linear fits for *Achillea*. ANOVA probabilities ($P > F$) of treatment effects and interactions: (a) *Lupinus*: CO_2 $P =$ **0.02**, N $P = 0.40(l)$, $P = 0.11(q)$; CO_2*N $P = 0.21(l)$, $P = 0.91(q)$; (b) *Achillea*: CO_2 $P = 0.14$; N $P <$ **0.0001** (l), $P =$ **0.003**(q); CO_2*N $P =$ **0.0009** (l), $P = 0.28(q)$.

Inc., Cary, NC). Correlation and regression are used for presentation, but we do not assume that direct causal relationships are involved.

Results

Plant growth

Lupinus total plant biomass increased on average 80% under elevated compared to ambient CO_2($P = 0.02$), whereas N additions did not affect biomass at either CO_2 concentration (Figure 1). In contrast, total plant biomass of *Achillea* increased to a greater extent with CO_2 enrichment under elevated than ambient N, ranging from no difference in low N soil to a 25% increase at the highest levels of N (CO_2 x N interaction $P = 0.0009$, Figure 1). Analyses of the allometric relations between shoots and roots, and nodules for *Lupinus*, indicated that there was no effect of elevated CO_2 on biomass distribution at final harvest (data not shown). Although elevated CO_2-grown plants had greater whole plant biomass than ambient CO_2-grown plants in both species, the increase in biomass was much greater in magnitude at these N levels for *Lupinus* than for *Achillea*.

Net photosynthesis

Mean rates of area-based net photosynthesis (A, μmol m^{-2} s^{-1}) in leaves grown under long-term elevated compared to ambient CO_2 concentrations (A_{700} vs. A_{365}, i.e. measured at growth CO_2 concentrations) were significantly higher in both species (+39% and +37% in *Lupinus* and *Achillea*, respectively, $P \leq 0.02$). These increases however, were smaller than the 70% average increase in A in response to short-term CO_2 enrichment [A_{365} (measured at 700) vs. A_{365}, Table 1, Figure 2]. This suggests that partial photosynthetic acclimation to elevated CO_2 occurred and was of similar magnitude in each species. Increases in A evaluated per unit leaf N (PNUE, μmol CO_2 gN^{-1} s^{-1}) also occurred to a similar magnitude in both species grown and measured under elevated compared to ambient CO_2 (average +60%, $P \leq 0.03$, data not shown). Significant CO_2-induced increases in achieved A in both species grown at elevated CO_2, were the result of both decreased leaf area per unit mass (SLA, cm^{-2} g^{-1}) and increased photosynthetic rates per unit leaf mass (A_{mass}, nmol g^{-1} s^{-1}, Table 1).

N addition had no effect on A in *Lupinus* plants (Table 1, Figure 2a) but *Achillea* showed 17% higher rates under the +16 gN m^{-2} yr^{-1} treatment compared with the controls (Figure 2b). Whereas N addition resulted in increased A in *Achillea*, it did not affect the response of A to elevated CO_2 (CO_2*N interaction $P = 0.95$). Thus, the increased N availability did not appear to affect the magnitude of photosynthetic acclimation to elevated CO_2 in either species.

Whole plant N concentration and plant N sources

Across the wide range of N additions, *Lupinus* whole plant N concentration (%) did not significantly change with either CO_2 enrichment or N addition (Figure 3, Table 2). In contrast, *Achillea* whole plant N concen-

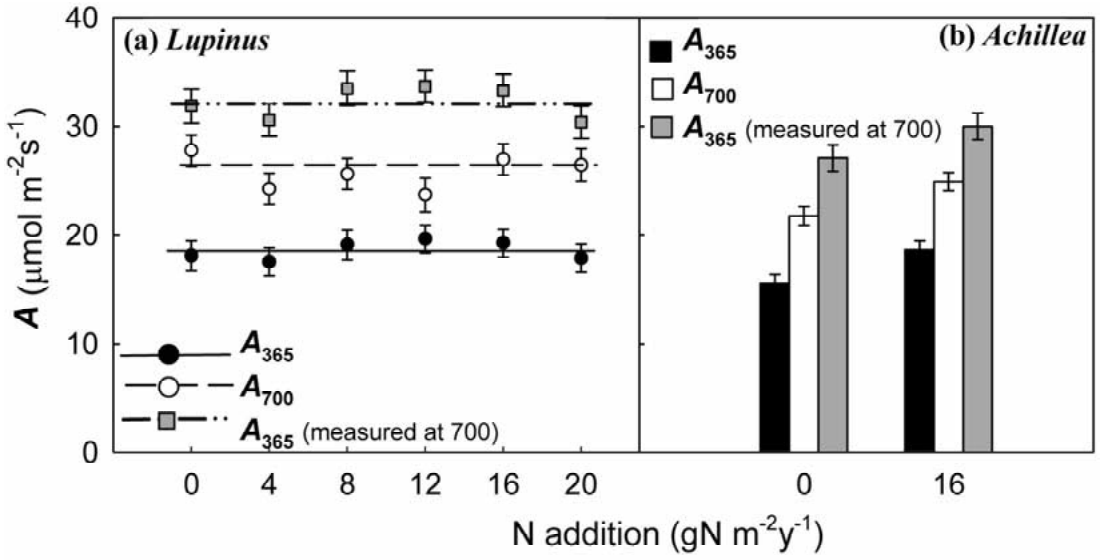

Figure 2. Light-saturated net photosynthetic rates per unit leaf area (A, μmol m^{-2} s^{-1}) of (a) *Lupinus* and (b) *Achillea* plants grown and measured in ambient (black circles/bars, 365 μmol mol^{-1}, A_{365}) and elevated (white circles/bars, 700 μmol mol^{-1}, A_{700}) CO_2concentrations, and ambient CO_2 grown plants measured at elevated CO_2 [gray squares/bars, A_{365} (measured at 700)]. Measurements were completed at (a) all levels of N addition or (b) +0 and +16 g N m^{-2} yr^{-1}. Least squares means (±SE, $n = 8 - 10$) from CO_2*N interaction, pooled across time, are shown. Horizontal lines mark the CO_2 treatment means in (a). Repeated measures ANOVA probabilities ($P > F$) for ambient grown plants exposed to short-term CO_2 enrichment: (a) measurement CO_2 $P < \mathbf{0.0001}$, N $P = 0.75(l)$, $P = 0.20(q)$, interactions n.s.; (b) measurement CO_2 $P < \mathbf{0.0001}$, N $P = \mathbf{0.04}$, interactions n.s. All other ANOVA probabilities ($P > F$) are shown in Table 1.

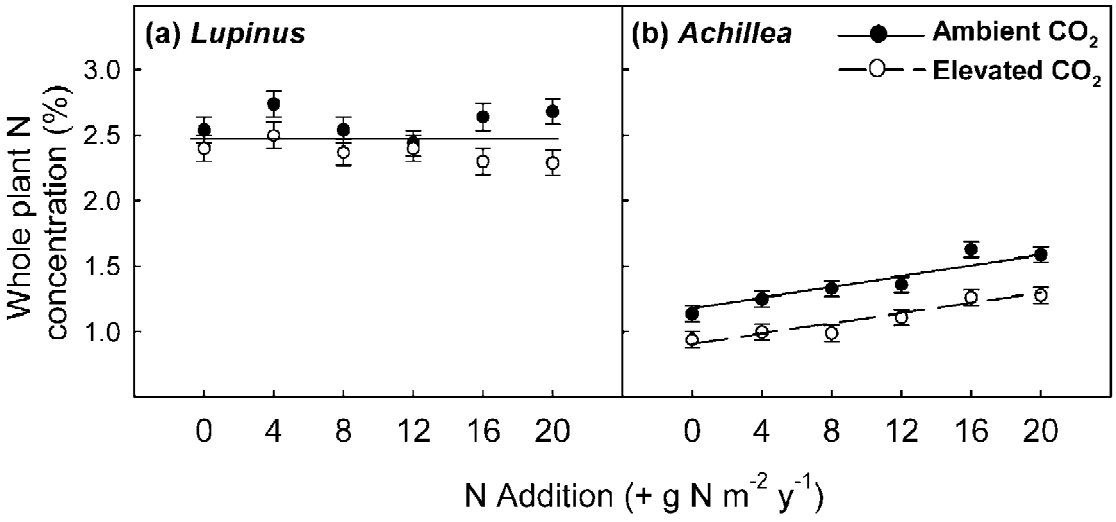

Figure 3. Whole plant N concentration (%) of (a) *Lupinus* and (b) *Achillea* after 56 days at ambient (•, 365 μmol mol^{-1}) and elevated (◯, 700 μmol mol^{-1}) CO_2 and varying levels of N addition. Least squares means (±SE, $n = 8 - 10$) from CO_2*N interaction are plotted with an overall mean line for *Lupinus* and CO_2*N linear fits for *Achillea*. ANOVA probabilities ($P > F$) are shown in Table 2.

Table 1. ANOVA probabilities ($P > F$) of the main effects of CO_2, N, and CO_2*N interaction on leaf net photosynthesis and related parameters of *Lupinus* and *Achillea* grown under ambient (365 μmol mol^{-1}) and elevated (700 μmol mol^{-1}) CO_2 concentrations and varying levels of N addition

Species	Response	CO_2	Nitrogen[1]		CO_2 * N	
			Linear	Quadratic	Linear	Quadratic
Lupinus perennis	A_{700} vs. A_{365}	**0.02** (+39%)[2]	0.79	0.60	0.76	0.06
	A_{365}(measured at 700) vs. A_{700}	**0.03**(−20%)	0.98	0.96	0.95	**0.04**
	A_{mass}	0.10(+14%)	0.87	0.51	0.73	0.08
	SLA	0.11 (−10%)	0.55	0.83	0.83	0.32
Achillea millefolium	A_{700} vs.A_{365}	**0.01** (+37%)	**0.004**(+17%)		0.95	
	A_{365}(measured at 700) vs. A_{700}	**0.04** (−18%)	**0.02** (+12%)		0.91	
	A_{mass}	0.88 (+2%)	0.13 (+12%)		0.15	
	SLA	0.13 (−24%)	0.62 (−2%)		**0.03**	

[1]For N treatments effects on *Lupinus*, orthogonal linear and quadratic contrasts are shown.
[2]$P \leq 0.05$ are bold-faced. Magnitude of main effects is shown as a percent change (elevated vs. ambient). A_{700}, A_{365} – net leaf photosynthesis on an area basis measured at growth CO_2 concentrations (μmol m^{-2} s^{-1}), A_{365}(measured at 700), $A_{700} - A$ measured at common CO_2 concentration of 700 μmol mol^{-1} (μmol m^{-2} s^{-1}), $A_{mass} - A$ on a mass basis measured at growth CO_2 concentrations (nmol g^{-1} s^{-1}), SLA – specific leaf area (cm^2 g^{-1}) were analyzed by repeated measures analysis of variance.

Table 2. ANOVA probabilities ($P > F$) for the main effect of CO_2, N, and CO_2*N interaction on whole plant N concentration, total plant N partitioned into originating sources (soil, fertilizer, symbiotic N_2 fixation), and the proportion of N derived from fixation

Variable	CO_2	*Lupinus* ($P > F$)				CO_2	*Achillea* ($P > F$)			
		N[1]		CO_2 * N			N		CO_2 * N	
		Linear	Quadratic	Linear	Quadratic		Linear	Quadratic	Linear	Quadratic
Whole plant N concentration (%)	0.10	0.49	0.79	0.25	0.34	**0.04**	<**0.0001**	0.59	0.26	0.60
Total plant N (mg plant^{-1})	**0.008**	0.73	0.14	0.70	0.30	0.37	<**0.0001**	0.79	0.09	0.46
Soil N (mg plant^{-1})	**0.50**	<**0.0005**	**0.0001**	**0.04**	**0.0002**	0.73	0.35	0.25	**0.02**	0.32
Fertilizer N (mg plant^{-1})	**0.01**	<**0.0001**	**0.0005**	**0.001**	0.70	0.59	<**0.0001**	**0.0003**	0.24	0.72
N_2 Fixation (mg plant^{-1})	**0.009**	<**0.0001**	**0.01**	0.68	**0.05**	NA[2]	NA	NA	NA	NA
Proportion of N derived from fixation (mg N_{fixed} (mgN plant)$^{-1}$)	**0.02**	<**0.0001**	<**0.0001**	**0.04**	<**0.0001**	NA	NA	NA	NA	NA

[1]For N treatments, orthogonal linear and quadratic contrasts are shown.
[2] Not applicable for non-fixing species.
[3]$P \leq 0.05$ are bold-faced.

tration decreased substantially in elevated compared to ambient CO_2 grown plants (−20%, $P = 0.04$) and N concentration increased progressively with increasing N addition ($P < 0.0001$, Figure 3, Table 2). These effects of CO_2 and soil N addition on plant N concentration were statistically independent in both species.

Figure 4 and Table 2 illustrate the effects of CO_2 and soil N addition on the amount of plant N at final harvest coming from the available sources: soil,

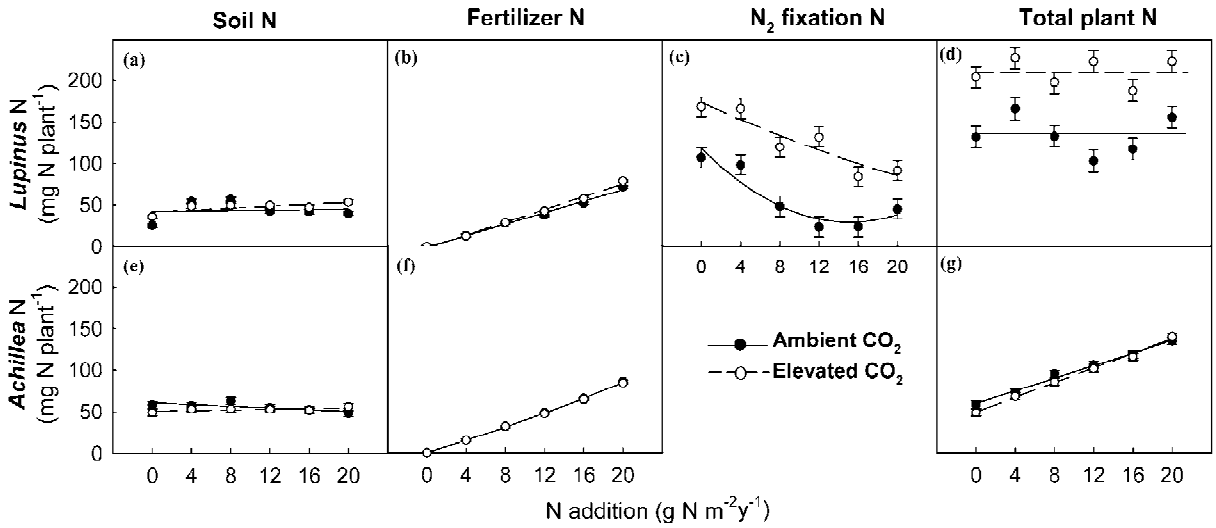

Figure 4. Total plant N (mg N plant^{-1}) partitioned into originating sources (soil, fertilizer, symbiotic N_2 fixation) for *Lupinus* (a–d) and *Achillea* (e–g) grown at ambient (•, 365 μmol mol^{-1}) and elevated (◯, 700 μmol mol^{-1}) $CO_2$and varying levels of N addition. Least squares means (±SE, $n = 8$) from CO_2*N interaction are plotted with linear or quadratic fits to the means based on ANOVA results shown in Table 2.

fertilizer, and symbiotic atmospheric N_2 fixation. In *Achillea*, total plant N increased linearly with increasing N addition levels due to the uptake of fertilizer N, whereas soil N uptake declined under ambient CO_2 and remained constant at elevated CO_2 (Figure 4e–g). Growth under elevated CO_2 had no apparent effect on total plant N or the proportions derived from soil compared to fertilizer in *Achillea* plants (Table 2, Figure 4e–g). In contrast, *Lupinus* plants increased the amount of total plant N when grown under elevated CO_2, and this increase came predominately from symbiotic N_2 fixation (on average + 120%, Table 2, Figure 4). With increasing N addition, the amount of N derived from N_2 fixation decreased in *Lupinus* (Figure 4c). This reduction in the amount of plant N coming from N_2 fixation was apparently compensated for by an increased uptake from soil plus fertilizer at low N supply rates and by increased uptake from fertilizer at higher N supply rates such that the total N of *Lupinus* was not greatly affected by N addition (Figure 4).

Not only did a greater total amount of N come from symbiotic N_2 fixation in elevated compared to ambient CO_2-grown *Lupinus*, but the proportion of plant N derived from symbiotic N_2 fixation increased 47% on average ($P = 0.04$). However, this stimulation of N_2 fixation was not independent of N supply rates (Figure 5, Table 2). The elevated CO_2-induced increase in the proportion of N derived from symbiotic fixation grew markedly with N supply level from 0 to 12 g N

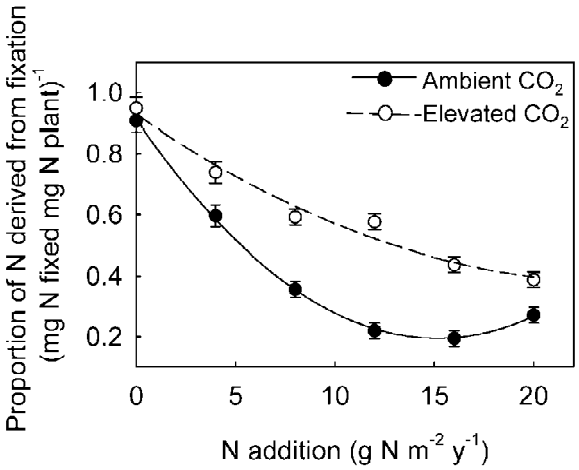

Figure 5. Proportion of N derived from symbiotic N_2 fixation [mg N fixed (mg N plant)$^{-1}$] in *Lupinus* grown under ambient (•, 365 μmol mol^{-1}) and elevated (◯, 700 μmol mol^{-1}) CO_2concentration and varying N addition levels. Least squares means (±SE, $n = 8 - 10$) from CO_2*N interaction are plotted with quadratic fits ($r^2 \geq 0.96$) to the means based on ANOVA results shown in Table 2.

m^{-2}y^{-1} but then diminished at yet higher N supply rates (Figure 5). Overall, the proportion of N coming from fixation declined with increasing N addition (Table 2, Figure 5). N_2 fixation values derived by the ^{15}N isotope method were generally comparable with estimates calculated by the difference method (Lee, 2001).

Discussion

N-limitations on the CO_2 response of Lupinus *and* Achillea

Growth of *Achillea* depended on N availability under both ambient and elevated CO_2 conditions. Also, low soil N limited the magnitude of the growth response of *Achillea* to elevated CO_2. These results support the hypothesis that low N limits the growth response of wild plant species to CO_2 enrichment. In contrast, the N_2-fixer *Lupinus* did not show evidence of N-limited growth. At these N levels, *Lupinus* demonstrated larger growth responses than *Achillea* to CO_2 enrichment that were independent of soil N supply. The lack of an N treatment effect on the *Lupinus* growth response to CO_2 enrichment is most simply explained by the consequences of the ability to access atmospheric N_2 through symbiotic fixation. *Lupinus* plants were similar in size, N concentration and in physiology across a wide range of soil N supply rates, and hence should not necessarily differ in response to elevated atmospheric CO_2.

While some simulation models suggest that photosynthetic responses and productivity under CO_2 enrichment are constrained at low N availabilities (Rastetter et al., 1997), evidence remains mixed (Poorter, 1998; Reich et al., 2001a,b). In a review by Lloyd and Farquhar (1996), average proportional plant growth enhancements due to elevated CO_2 were even greater under low than high N conditions in some cases. Several studies have found that despite significant effects on plant growth, nutrient addition did not enable greater responses to elevated atmospheric CO_2 (Hättenschwiler and Körner, 1996; Körner et al., 1997; Reich et al., 2001a,b). In contrast, other studies found an increasing magnitude of growth stimulation in response to elevated CO_2 as N supply increased (Curtis et al., 2000; Wand et al., 1999).

Although partial photosynthetic acclimation to CO_2 enrichment occurred, both species maintained significantly higher rates of photosynthesis (+38%, see Table 1) and more efficient carbon capture per unit leaf N (average +60%, data not shown) in elevated compared to ambient CO_2, which is comparable with many, but not all, lab-based studies (Tjoelker et al., 1998; Wand et al., 1999; but not Sims et al., 1998). The down-regulation of photosynthesis under elevated CO_2 is likely explained in part by decreased tissue N concentrations and in part by several additional changes in leaf form and physiology, as observed in many other studies (e.g., Lee et al., 2001; Tjoelker et al., 1998). In addition, in contrast to the biomass response, the photosynthetic response to CO_2 enrichment did not depend on soil N supply in either species. Our results, therefore, do not support the hypothesis that leaf-level photosynthetic response to elevated CO_2 is limited at low N, nor that N_2-fixing species will maintain higher photosynthetic enhancements with CO_2 enrichment than non-fixing species. The literature remains split between those that also found enhancements in photosynthetic rates of plants with CO_2 enrichment independent of nutrient availabilities (Hättenschwiler and Körner, 1996; Körner et al., 1997; Lloyd and Farquhar, 1996; Lee et al., 2001) and those that found elevated CO_2-induced photosynthetic enhancements to be significantly greater at higher compared to low nutrient availability (Curtis et al., 2000; Sims et al., 1998; Ward and Strain, 1999). The differential growth responses of *Lupinus* and *Achillea* to the combination of CO_2 and N in this study likely involve the integration of leaf-level and/or plant-level gas exchange and morphological or allocational changes in response to elevated CO_2 (Ward and Strain, 1999).

Relationships between functional traits and species response to elevated CO_2 and increasing N

The differential growth responses *Lupinus* and *Achillea* to elevated CO_2 support the hypothesis that biomass stimulation under elevated CO_2 is stronger in N_2-fixing than non-fixing species, because the former are not N-limited. Even at the highest levels of soil N availability, the response of *Achillea* to elevated CO_2 remained significantly less than that of *Lupinus*. Perhaps this was because neither the N response nor the N effect on the elevated CO_2-induced response of *Achillea* appeared to reach saturation even at the highest levels of N (Figure 1) suggesting that further increases in the responsiveness of *Achillea* to elevated CO_2 might occur. However, the range of N amendments supplied were up to eight times the background supply of N in this N-poor soil and N supply rates greater than 20 g m^{-2} y^{-1} are highly unlikely under natural conditions. In a review of responses to elevated CO_2 under a variety of soil nutrient conditions including 156 species (Poorter, 1993), legume species demonstrated an average 50% increase in biomass, whereas other C_3 species biomass increased 41%. Stronger legume growth responses compared to non-legumes have also been found in several other

studies (e.g., Hebeisen et al., 1997a,b; Lüscher et al., 1996; Soussana and Hartwig, 1996) but not others (Leadley and Körner, 1996 or Reich et al., 2001b).

With access to atmospheric N_2, N_2-fixers may be able to alleviate N-limitations on CO_2-induced stimulation of growth when non-fixers cannot (Lüscher et al., 1996, 2000). The additional N available through symbiotic N_2 fixation may increase both the capacity for enhanced rates of realized photosynthesis with CO_2 enrichment and the utilization of this additional photoassimilate, thus facilitating continued or greater growth stimulation by elevated CO_2 (Daepp et al. 2001; Poorter, 1993). *Lupinus* accumulated substantially more plant N and biomass than *Achillea* in response to growth at elevated CO_2 and maintained similar tissue N concentrations in elevated compared to ambient CO_2 plants, which likely helped minimize a more severe down-regulation of photosynthesis that would otherwise reduce elevated CO_2 induced enhancement of growth. In contrast, elevated CO_2 grown *Achillea* did not accumulate greater total amounts of N compared to plants grown at ambient CO_2, even when plants were larger, due to the significant reduction in tissue N concentrations (see Table 2 and Figure 3). Indirectly, this suggests that *Achillea* may not have had access to enough N or did not have sufficient sink demand (Daepp et al., 2001) to fully utilize the potentially available carbon under elevated CO_2, especially when grown at low N. Alternatively, *Achillea* plants may have grown larger with CO_2 enrichment even with apparent N-limitation owing to an increase in biomass produced per unit of N.

N derived from symbiotic N_2 fixation under elevated CO_2 and increasing N

The higher total plant N in elevated compared to ambient CO_2 grown *Lupinus* was derived predominantly from increased symbiotic N_2 fixation (Figure 4). In addition, the relative proportion of total plant N that was derived from fixation was on average 19 percentage points higher under elevated CO_2 compared to ambient CO_2(59% compared to 40%, respectively). However, the amount of N fixed per gram nodule mass was not affected by CO_2 concentration ($P = 0.64$, data not shown). Therefore, assuming nitrogenase content per gram nodule was the same, the increase in the proportion of N derived from fixation with CO_2 enrichment was a result of an increased number and overall mass of nodules, rather than changes in specific nitrogenase activity, in agreement with previous field and laboratory studies (Murphy, 1986; Soussana and Hartwig, 1996; Zanetti et al., 1996; Zanetti and Hartwig, 1997).

Comparable increases in the amount of N derived from symbiotic N_2 fixation at elevated compared to ambient CO_2 have been found in several studies, including those with herbage legumes in controlled chamber environments (e.g., Murphy, 1986), in greenhouses using soil monoliths (Soussana and Hartwig, 1996), and in field studies (Lüscher et al., 2000; Zanetti et al., 1996, 1998; Zanetti and Hartwig, 1997). Most also found that the relative proportions of total plant N coming from N_2 fixation compared to other sources of N increased. Some studies, however, have shown relatively minimal effect of CO_2 enrichment on the proportion of N derived from N_2 fixation (e.g., Zanetti et al., 1998). It is the increased demand for N in elevated CO_2-grown plants that appears to drive increases in overall N_2 fixation (Hartwig and Nösberger, 1994; Soussana and Hartwig, 1996; Zanetti al., 1996, 1998). Variations in the magnitude of this response likely correlate with the availability of other resources such as soil moisture, or levels of other nutrients like P, Mo, or Fe, which are critical for and often limit N_2 fixation (Almeida et al., 2000; Bordeleau and Prévost, 1994; Lüscher et al., 1996; Niklaus et al., 1998; Vitousek and Field, 1999).

Conclusions

The CO_2 response of wild species, even those adapted to low N habitats, can be limited by low N availability; however, N_2-fixers can abate this limitation by supplementing mineral N uptake with symbiotic N_2 fixation. This was apparent in the legume, *Lupinus*, grown under elevated CO_2 and increasing soil N addition. The stronger response of *Lupinus* compared to *Achillea* can be attributed to the increased demand for N under elevated CO_2, which could be met only in *Lupinus* through increased symbiotic N_2 fixation. Since this response could lead to modified roles of N_2-fixers and N_2-fixer/non-fixer species interactions in grassland communities, especially those that are inherently N-poor, under projected rising atmospheric CO_2, it is important to consider interactive effects of CO_2 and N availability, and the differences in species responses to combinations of these factors, when attempting to predict plant responses to the environments of the future.

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